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(71) Applicant *(for all designated States except US)*: BIOGEN, INC. [US/US]; 14 Cambridge Center, Cambridge, MA 02142 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : STAPLES, Mark, A. [US/US]; 12 Stoneholm Street, #612, Boston, MA 02115 (US). PARGELLIS, Christopher, A. [US/US]; 18 Limekiln Road, West Redding, CT 06896 (US).

(74) Agents: HALEY, James, F., Jr. et al.; Fish & Neave, 875 Third Avenue, New York, NY 10022 (US).

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(54) Title: PROCESS FOR PURIFYING A PROTEIN

(57) Abstract

A process for the purification of proteins from solutions containing contaminants of similar net charge and molecular weight is provided, comprising contacting a solution containing the desired protein with an immobilized metal affinity chromatography resin in a buffer containing a low concentration of a weak ligand for the chelant of the resin. The adsorbed protein is then eluted using a buffer having a high concentration of the same weak ligand, e.g., Tris. Particularly preferred features employ agarose-iminodiacetic acid resins having copper cations and are especially useful in obtaining preparations of homogeneous, stable rsT4 proteins.

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PROCESS FOR PURIFYING A PROTEIN

TECHNICAL FIELD OF THE INVENTION

This invention relates to a process for purifying proteins. More particularly, the invention relates to a process for purifying proteins containing surface metal-binding amino acid residues such as histidine and cysteine.

BACKGROUND

Various chromatography techniques are known in the art for purifying proteins. Procedures such as molecular sieve chromatography, ion exchange chromatography, and electrophoresis are commonly utilized to purify proteins. Separation of proteins that have very similar molecular weights and similar net charges, however, requires the use of alternative purification methods due to the absence of any significant differential in the features (i.e., molecular weight and net charge) which known separation processes exploit. Complete and efficient separation of proteins intended for therapeutic use is critical, particularly if the purified protein is to be used in the treatment of hypersensitive individuals such as immunodeficient or immunocompromised patients.

An alternative technique for purifying proteins under limited conditions has been termed

"Immobilized Metal Affinity Chromatography" (IMAC). The development of this method resulted from the recognition that certain proteins have an affinity for heavy metal ions, which could be an additional distinguishing feature to use in attempting separation of the proteins. This feature applies especially to proteins containing histidine or cysteine residues, which have been found to complex with chelated zinc or copper ions and become adsorbed on a chelating resin [J. Porath et al., "Metal Chelate Affinity Chromatography, A New Approach To Protein Fractionation", Nature, 258, pp. 598-99 (1975)].

A difficulty with the technique arises, however, in selectively desorbing the proteins from the resin. A common technique for desorption is lowering the pH to about 3 or 4 [A.J. Fatiadi, "Affinity Chromatography And Metal Chelate Affinity Chromatography", CRC Critical Reviews in Analytical Chemistry, 18, pp. 1-44 (1987)]. Another method consists of adding solutes to the eluant which have a stronger affinity than the proteins for binding to the chelated metal. This involves using strong complexing agents such as histidine or EDTA, which bind tightly to the metal [A. Figueroa et al., "High-Performance Immobilized-Metal Affinity Chromatography Of Proteins On Iminodiacetic Acid Silica-Based Bonded Phases", J. Chromatography, 371, pp. 335-52 (1986)].

With the latter technique, the metal is often stripped from the column; such "bleeding" of the metal ions is obviously an unwanted effect in a purification process. Figueroa et al. reported the use of ammonia, a weak competing ligand, as an eluant to desorb slightly bound proteins from an IMAC column. Their procedure, however, involved the use of HPLC and of a complex binding buffer system, requiring additional

washings of the column and switching to ammonia for elution. Both of these factors add to the time involved in running the purification and detract from the efficiency and yield of the purification process.

In addition, the commonly used technique of lowering the pH to desorb proteins from the column is generally effective only for desorption of strongly bound proteins, since low pH desorption often promotes non-selective desorption of all proteins.

Genetic engineering technology has made possible the production of recombinant proteins in quantities hitherto unavailable. However, these proteins often have major contaminants which have presented an obstacle in purifying the proteins into pharmaceutically acceptable form. Current purification procedures are only partially effective in the purification of proteins found with contaminant proteins having similar molecular weight and net charge. As a result there is a continuing need for methods for purifying such proteins so as to increase the availability of new therapeutic agents.

SUMMARY OF THE INVENTION

This invention solves the problems referred to above by providing a process for the purification, in high yield, of proteins containing surface metal-binding amino acid residues. In a particularly preferred embodiment, the process of this invention allows the purification of soluble T4 protein by selective desorption from a Cu^{2+}-IDA substrate using the same buffer for binding and for eluting the proteins from the substrate.

The present invention provides a method for separating proteins based on the nature and distribution of their constituent amino acids.

More particularly, the present invention provides a method for separating proteins according to the affinity of their constituent amino acid residues for specific metal cations.

The present invention further provides a method for separating proteins from contaminants of substantially similar molecular weight and net charge.

Thus, the present invention encompasses a process for purifying a protein having surface metal-binding amino acid residues by the steps:

(a) preparing an immobilized metal affinity chromatography (IMAC) resin comprising a matrix resin linked to a bidentate chelator having bound divalent metal ions (Me^{2+}), in a binding buffer containing a weak ligand for said metal ions, such as Tris, ammonia, and the like;

(b) contacting a solution containing the protein (which may also contain contaminant protein(s) or protein fragments of similar net charge and molecular weight) with the resin; and

(c) selectively eluting the protein using a buffer containing a higher concentration of the weak ligand than in the equilibration buffer.

As alluded to above, the present invention may be advantageously applied in the purification of a protein gaining particular interest in the investigation of acquired immune deficiency syndrome (AIDS), namely, soluble T4 (CD4).

T4 proteins serve as the receptors on the surface of $T4^+$ lymphocytes. In immunocompetent individuals, T4 lymphocytes interact with other specialized cell types of the immune system to confer immunity to or defense against infection [E.L. Reinherz and S.F. Schlossman, "The Differentiation And Function Of Human T Lymphocytes", Cell, 19, pp. 821-27 (1980)].

More specifically, T4 lymphocytes stimulate production of growth factors which are critical to a functional immune system. For example, they act to stimulate B cells, the descendants of hemopoietic stem cells, which promote the production of defensive antibodies. They also activate macrophages ("killer cells") to attack infected or otherwise abnormal host cells, and they induce monocytes ("scavenger cells") to encompass and destroy invading microbes.

The primary target of the receptor for certain infective agents is the T4 surface protein. These agents include, for example, viruses and retroviruses. When T4 lymphocytes are exposed to such agents, they are rendered nonfunctional. As a result, the host's complex immune defense system is destroyed and the host becomes susceptible to a wide range of opportunistic infections.

Such immunosuppression is seen in patients suffering from acquired immune deficiency syndrome ("AIDS"). Complete clinical manifestation of AIDS is usually preceded by AIDS related complex ("ARC"). The human immunodeficiency virus ("HIV") is thought to be the etiological agent responsible for AIDS infection and its precursor, ARC [M.G. Sarngadharan et al., "Antibodies Reactive With Human T-Lymphotropic Retroviruses (HTLV-III) In The Serum Of Patients With AIDS", Science, 224, pp. 506-08 (1984)].

The host range of HIV is associated with cells which bear the surface glycoprotein T4. The tropism of HIV for $T4^+$ cells is attributed to the role of the T4 cell surface glycoprotein as the membrane-anchored virus receptor. Because T4 behaves as the HIV receptor, its extracellular sequence probably plays a direct role in binding HIV. A cloned cDNA version of human T4, when expressed on the surface

of transfected cells from non-T cell lineages, including murine and fibroblastoid cells, endows those cells with the ability to bind HIV [P.J. Maddon et al., "The T4 Gene Encodes The AIDS Virus Receptor And Is Expressed In The Immune System And The Brain", Cell, 47, pp. 333-48 (1986)].

Therapeutics based upon soluble T4 protein have been proposed for the prevention and treatment of the HIV-related infections AIDS and ARC. The nucleotide sequence and a deduced amino acid sequence for a DNA that purportedly encodes the entire human T4 protein have been reported [P.J. Maddon et al., "The Isolation And Nucleotide Sequence Of A cDNA Encoding The T Cell Surface Protein T4: A New Member Of The Immunoglobulin Gene Family", Cell, 42, pp. 93-104 (1985)]. The amino acid sequence is depicted in Figure 1 herein. Based upon its deduced primary structure, the T4 protein is divided into the following domains:

Structure/Proposed Location	Amino Acid Coordinates
Hydrophobic/Secretory Signal	-23 to -1
Homology to V-Regions/ Extracellular	+1 to +94
Homology to J-Regions/ Extracellular	+95 to +109
Glycosylated Region/ Extracellular	+110 to +374
Hydrophobic/Transmembrane Sequence	+375 to +395
Very Hydrophilic/ Intracytoplasmic	+396 to +435

Soluble T4 proteins have been constructed by truncating the full length T4 protein at amino acid

375, to eliminate the transmembrane and cytoplasmic domains. Such proteins have been produced by recombinant techniques [R.A. Fisher et al., "HIV Infection Is Blocked In Vitro By Recombinant Soluble CD4", Nature, 331, pp. 76-78 (1988)]. Soluble T4 proteins advantageously interfere with the T4/HIV interaction by blocking or competitive binding mechanisms which inhibit HIV infection of cells expressing the T4 surface protein. Soluble T4 proteins inhibit interaction between $T4^+$ lymphocytes and antigen presenting cells and targets of $T4^+$ lymphocyte mediated killing. By acting as soluble virus receptors, soluble T4 proteins are useful as anti-viral therapeutics to inhibit HIV binding to $T4^+$ cells and virally induced syncytium formation.

Thus, recombinant soluble T4 protein (rsT4), due to its activity as the HIV receptor, can be effective in the treatment of AIDS, ARC, HIV infection and other immunodeficiencies caused by T4 lymphocyte depletion or abnormalities. It is therefore desirable to produce pure forms of soluble T4 in large amounts for clinical and therapeutic uses. Particularly when the protein is to be injected into the bloodstream of immunodepressed individuals, it must be free of toxic contaminants. In order to meet this demand, the need exists for a purification method which allows efficient preparation of rsT4 free of contamination by destabilizing or toxic factors.

The soluble T4 prepared by current techniques, however, is accompanied by a contaminant (fragment Bb of complement factor B) having a similar molecular weight and charge. Thus, prior methods such as molecular sieve chromatography, ion exchange chromatography, and electrophoresis are not feasible for complete purification.

A preferred protein purified according to the process of this invention is recombinant soluble T4, the receptor on the surface of $T4^+$ lymphocytes. The purified, stable rsT4 produced according to this invention is useful in treating immunodeficient patients suffering from diseases caused by infective agents whose primary targets are $T4^+$ lymphocytes. More particularly, the soluble T4 protein purified according to the process of this invention is useful in preventing, treating or detecting acquired immune deficiency syndrome, AIDS related complex (ARC), and HIV infection.

A particular object of this invention is to provide purified, homogeneous, recombinant soluble T4 protein in a stable conformation, which may, in turn, be used in the treatment or prevention of AIDS, ARC, and HIV infection.

In a preferred embodiment, therefore, the invention comprises the steps of (a) contacting a solution containing rsT4 protein, preferably free of source cells and cellular debris, with an immobilized metal affinity chromatography (IMAC) resin comprising a matrix resin linked to a bidentate chelator bound to divalent metal ions, in a binding buffer containing salt, such as NaCl or KCl (preferably NaCl), and a weak ligand for said metal ions, and (b) selectively eluting the rsT4 protein using a buffer containing salt and a higher concentration of the weak ligand than in the binding buffer. Preferably, the concentration of salt in the elution buffer will be about the same as that of the binding buffer, and preferably the concentration of the weak ligand in the elution buffer will be about 10-50 times that of the binding buffer. Preferred matrix resins are agarose gels.

It is also preferred that solution components which may be purified by conventional means will be eliminated from the sample prior to the application of IMAC resin according to this invention. Therefore, in another preferred embodiment, the process of this invention comprises the steps: (1) contacting the culture medium containing the rsT4 protein, filtered free of the source cells which produced the rsT4, with a cationic exchange resin, which adsorbs the protein; (2) eluting the adsorbed proteins from the resin on the basis of their net charge; (3) applying the fraction or fractions of eluate from the cationic exchange resin (which will contain the rsT4 protein) to an anionic exchange resin which adsorbs contaminants while allowing rsT4 and other proteins with similar pI values to wash through without binding to the resin; (4) applying the "wash" fraction or fractions containing rsT4 to an immobilized metal affinity chromatography (IMAC) resin, prepared, e.g., by suspending a gel comprised of agarose and a bidentate chelator, e.g., iminodiacetic acid, in a solution of a metal salt, using a binding buffer containing salt (e.g., NaCl or KCl) and a low concentration (e.g., 0.01 to 0.05 M) of a weak ligand for the metal ion of the metal affinity chromatography resin; and (5) eluting the rsT4 using as an eluant a buffer having the same salt concentration but a higher concentration (e.g., 0.1 to 0.5 M) of the same weak ligand.

In the foregoing scheme, preferably the metal salt used in preparing the metal affinity resin will be $CuCl_2 \cdot 2H_2O$; preferably the binding buffer will contain Tris·HCl as the weak ligand in a concentration of about 0.01 to 0.1 M, most preferably about 0.02 M; preferably the salt of the binding and elution buffers will be NaCl or KCl in a concentration (in both

buffers) of about 0.1 to 1.0 M; and preferably the elution buffer will contain Tris·HCl in a concentration of about 0.1 to 0.5 M, most preferably about 0.3 M. A gradient of Tris buffer may also be used in elution. Furthermore, as used herein, the term "weak ligand" is defined with reference to the protein to be purified (e.g., rsT4) and the particular metal affinity resin employed. The weak ligand will have a lesser affinity for the binding resin than the protein to be separated. In general, the most preferred weak ligands will be ammonia or organic amines.

The resulting purified rsT4 obtained as above can be used therapeutically to prevent, treat, or detect acquired immune deficiency syndrome (AIDS), AIDS related complex (ARC), and HIV infection.

This method of preparation and purification is also useful for purifying other proteins having sufficient surface metal binding amino acid residues to bind to an IMAC column, and which are desorbed from the column with a weak competing ligand, such as Tris·HCl.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 depicts the amino acid sequence of natural T4 (CD4) protein (a transmembrane protein), as reported by Maddon et al., "The Isolation And Nucleotide Sequence Of A cDNA Encoding the T Cell Surface Protein T4: A New Member Of The Immunological Gene Family", Cell, 42, pp. 93-104 (1985).

Figure 2 depicts the amino acid sequence of a rsT4 protein which may be advantageously purified according to the process described herein.

Figure 3 is the amino acid sequence of fragment Bb from complement Factor B.

Figure 4 is a schematic representation of an immobilized metal affinity resin useful in the process of the invention.

The process according to the invention will now be described in further detail, using the purification of a recombinant soluble T4 protein as a specific example. While the following description relates to a particular protein advantageously purified according to the invention, it will be understood that the process as described will be applicable to the separation of a wide variety of other proteins having surface metal-binding amino acids, which proteins will be immediately recognized by those skilled in this art. The following description is not intended to limit the scope of the invention in any way.

DETAILED DESCRIPTION OF INVENTION

The present invention provides a process for the purification of recombinant soluble T4 and other proteins bearing surface metal-binding amino acid residues. The process of the invention provides the protein in a stable, native conformation suitable for administration to humans.

The following discussion refers to the separation of a rsT4 protein, however the principles disclosed will be readily applicable to the purification of other proteins containing metal-binding amino acid residues, for separation from other proteins or protein fragments of similar molecular weight and charge.

A crude sample containing the rsT4 protein is obtained, for example, by suspending and incubating cells containing the gene coding for rsT4 in a cell growth medium containing collagen beads, then filtering the medium to remove the source cells. The process of

the invention may include the initial step of contacting the sample containing the rsT4 with a cationic exchange resin. It is preferred that an anionic gel with a capacity of about 20 meq/ml gel or greater and a fast flow rate suitable for large-scale purification be used. Most preferably, the gel used is S-Sepharose Fast Flow™, (Pharmacia LKB), which uses a bound anionic sulfate ligand and a maximal linear flow rate of 400 cm/h. By adjusting the initial pH to about 5.5, many proteins in the sample, including the rsT4 protein, are positively charged and will become adsorbed on the resin. The flow-through contains non-adsorbing contaminants which may be discarded. The resin can then be washed with a buffer at an increased pH and with an increased salt concentration. Any buffer with a pKa in the pH range of about 2-10 may be used. Preferably, the elution buffer is Tris·HCl at pH 8.5 and contains 0.05 to 1.0 M, preferably 0.1 M, concentration of a salt which is preferably NaCl. Rinsing the resin with this buffer causes desorption and elution of most of the proteins contained in the original crude sample.

After washing the cationic exchange resin with the buffer solution, the fraction or fractions of eluate containing rsT4 protein may be contacted with an anionic exchange resin. Preferably such resin will have a capacity of 20 meq/ml gel or greater and a flow rate suitable for large-scale purification. Most preferably, the gel used is Q-Sepharose Fast Flow™ (Pharmacia LKB), which uses the cation of the diethyl(2-hydroxy-propyl) aminoethyl group and a maximal linear flow rate of 400 cm/h. Since the pH of the eluate from the cationic exchange step is about 8.5, all proteins except those with a pI in the basic range (i.e., pH above 8.5) will be negatively charged

and will be adsorbed onto the resin. The flow-through will contain those proteins with a pI above 8.5, including the rsT4. This step results in approximately a 70% yield of 90% pure rsT4.

This step is particularly suited for proteins such as rsT4 which contain a large number of basic amino acid residues such as lysine, arginine and histidine. The rsT4 sequence depicted in Figure 2, for example, contains 38 lysine residues, which makes the overall pI of the molecule unusually basic. For this reason, the use of a high pH in the anion exchange step successfully separates the rsT4 and similar proteins from the large number of protein contaminants which have a lower lysine content and consequently have pI values much closer to the norm of about 7.5. After obtaining the flow-through from the anion exchange step, major contaminants remaining are proteins (or protein fragments) also having a high number of basic residues and consequently basic pI values. In the case of rsT4, a major contaminant that remains in the solution is fragment Bb of complement factor B (see Figure 3).

For the next step in the purification, the eluate from the anion exchange step is adjusted from pH 8.5 to pH about 7.5 with the addition of acid. Although any acid may be used to lower the pH, the acid used in this technique is preferably HCl. The salt (NaCl) concentration is also increased, preferably to 0.15 M. The preparation is then directly contacted with a metal chelating resin containing bound metal cations. Any resin which can be linked with a chelating agent may be used to form the matrix for the metal cations. The preferred "matrix resin" is Chelating Sepharose 6B™ (Pharmacia), an agarose-based resin which is linked to iminodiacetic acid (IDA), with

a dicarboxylic acid group which serves as a bidentate chelator. Other resins which are inert to the solutions they are contacted with and which are capable of acting as a substrate for bidentate chelator molecules, such as iminodiacetic acid, are also suitable. Examples include dextran, crosslinked acrylamide, beaded cellulose, and the like. The resin is contacted with a divalent metal ion in order to cause chelation, or immobilization of the ion, as shown schematically in Figure 4.

The divalent metal ion (Me^{2+} in Figure 4) is chosen from, but not limited to, the alkaline earth metals and the first row transition metals having atomic numbers ranging up to 30. Preferably, the divalent metal cations will be selected from the group containing Nickel(II), Zinc(II), Cobalt(II), and Copper(II). The preferred metal for the agarose-IDA resin of this embodiment is Cu^{2+} due to its stronger binding constants with IDA and with rsT4. When the flow-through from the anion exchange step is contacted with this IMAC resin, the exposed histidine residues on the proteins will bind to the immobilized metal. The rsT4 and the fragment Bb both contain histidine residues and will thus bind to the resin. The rsT4 protein, however, contains only 4 histidine residues, whereas its major contaminant, fragment Bb of complement factor B, has 13 histidine residues (cf. Figure 2; Figure 3).

As can be seen from the foregoing discussion, the number of histidine residues in a protein is a primary indicator of binding strength between the protein and the resin. However, the strength of protein-resin binding will depend on the accessibility of histidine residues to the resin and on the proximity of multiple histidine residues.

Therefore, unless the 3-dimensional configuration of the protein is known, some experimentation will be required to estimate the protein-resin binding strength and the suitability of this invention for increasing the purity of a protein preparation.

In order to selectively elute the rsT4 from the resin, a buffer must be chosen which will compete with the proteins for binding to the metal on the basis of the strength of the protein's affinity for the copper. Buffers with a stronger affinity for the metal than the protein's affinity for the metal would not be effective in selective desorption as they would disrupt all His-Cu^{2+} binding and desorb all bound proteins simultaneously. Weaker ligands such as Tris (tris(hydroxymethyl)methylammonium^{+}), would not be expected to cause desorption since the affinity of the histidine residues on the protein for the metal is much stronger than the Tris molecule's affinity, probably due in part to the much more effective electron donation of the imidazole ring of histidine relative to the lone pair of electrons of the Tris nitrogen, and to the multiple-point attachment of the protein to the resin.

Surprisingly, however, continuing the use of Tris·HCl at an increased concentration, e.g., of 0.3 M, causes sufficient saturation and competition with the rsT4's His-Cu^{2+} binding to allow desorption of the rsT4 from the resin. The contaminant, however, remains adsorbed on the resin. Other weak ligands such as ammonia will also be effective in this step to accomplish desorption. Use of a different weak ligand as the eluant, however, decreases the efficiency of the process due to the need to change buffer systems to ammonia for elution.

This purification step removes the final major contaminant from the rsT4 and results in a greater than 60% yield of rsT4 which is of greater than 94% purity.

The rsT4-containing eluate from the immobilized Cu^{2+} resin may be concentrated by precipitation with ammonium sulfate; and as a further preferred step of this purification, the rsT4-containing precipitate is dissolved, preferably in phosphate buffered saline (PBS), and the solution applied to a Sephacryl S100 HR™ (Pharmacia) column to separate on the basis of molecular size. Although the Sephacryl S100 HR™ resin is preferred, any size-exclusion resin which separates molecules having a molecular weight in the range of (in this instance, for rsT4) 40,000 may be used in this step. Other solutions or materials may also be employed for redissolving the precipitate or as the elution buffer in this application of size-exclusion chromatography; and such alternative solutions or materials will normally be selected with an eye to the desired final formulation and whether the protein is being prepared, e.g., for storage or immediate administration to patients or some other end use. One such alternative material is glycine (e.g., about 0.5% w/v). In another mode, the purified protein may be resuspended in phosphate buffer (PBS) alone or phosphate buffer having, e.g., about 5.0% w/v mannitol.

The pure, stable rsT4 preparation obtained as above can be diluted to the appropriate dosage strength and used directly in the treatment of immunodeficient and immunocompromised patients.

The method of this invention may be utilized for purifying any protein which contains enough metal-binding residues such as histidine or cysteine to

allow binding to an immobilized metal ion chelating resin. Such proteins may be selectively desorbed from the resin using a weak competing ligand, such as Tris, for separation from similarly charged and sized contaminant proteins or protein fragments which have a differential composition or distribution of surface histidine and cysteine residues, as compared with the protein to be separated.

The following examples of the purification of rsT4 protein are set forth by way of illustration of the process according to the present invention and are in no way intended to limit the scope of the teaching of applicants' invention.

EXAMPLES

Example 1

PARTIAL PURIFICATION OF RECOMBINANT SOLUBLE T4 VIA CATION-EXCHANGE CHROMATOGRAPHY

We first obtained a 400 L sample containing rsT4 protein by suspending and incubating cells containing the gene coding for rsT4 (CHO Clone 6, provided by Biogen, Inc., Cambridge, MA) on collagen beads in a bioreactor. The rsT4 is secreted as a soluble protein into the extracellular medium. We then removed the medium from the bioreactor and subjected it to 0.2 micron ultrafiltration to remove the source cells. Next, we diluted this sample with an equal volume of water and adjusted the pH to 5.5 by adding 1% acetic acid. We then loaded this solution onto a 4.0 L column with a height of 6.5 cm which contained S-Sepharose, a cation exchange resin in a ratio of 140 mg protein/ml gel. We washed with 7.5 column volumes of 0.015 Tris·HCl buffer at pH 8.5. We then washed the column with 1.7 column volumes of 0.015 M Tris·HCl buffer at pH 8.5, containing 0.1 M NaCl, to elute the

adsorbed proteins. This step produced rsT4 protein of about 60% purity.

FURTHER PURIFICATION OF RECOMBINANT SOLUBLE T4 VIA ANION-EXCHANGE CHROMATOGRAPHY AT HIGH pH

We then loaded the fractions of eluate containing rsT4 from the cation exchange column, diluted with 1.1 volumes of Tris·HCl and adjusted to pH 8.5, directly onto a 2.5 L column containing Q-Sepharose Fast Flow™, an anion exchange resin, in a ratio of 10 mg protein/ml gel. We collected the flow-through from the column and then regenerated the resin for further use. The flow-through afforded rsT4 in about a 70% yield with a 90% purity level.

PREPARATION OF IMMOBILIZED METAL ION COLUMN

First, we washed one column volume of Chelating Sepharose 6B™ gel with several column volumes of water. We then suspended the washed gel in four volumes of 0.05 M $CuCl_2 \cdot 2H_2O$ for a period of at least 30 minutes. We then washed the gel again with several volumes of water to remove any uncomplexed Cu^{2+}. Finally, we equilibrated the gel by washing it with several volumes of 0.02 M Tris·HCl buffer (pH 7.5), containing 0.15 M NaCl.

FURTHER PURIFICATION OF rsT4 BY IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY

After pooling the flow-through from the anion exchange chromatography step, we adjusted the pH of the sample to 7.5 by addition of HCl (1.0 M) and increased the salt concentration to 0.15 M by addition of NaCl. We then applied the flow-through pool to a 2.5 L column containing the immobilized copper resin. The sample was loaded onto the column in a ratio of 10

mg protein/ml gel and at a temperature of 4°C. The flow rate for the column was 6 column volumes per hour.

The loaded column was then washed with several column volumes of 0.02 M Tris·HCl buffer (pH 7.5) containing 0.15 M NaCl to allow binding of proteins and flow-through of non-binding contaminants. The column effluent was fed through a UV spectrophotometer, and the rsT4 was eluted from the column by washing with 0.2 M Tris·HCl (pH 7.5) containing 0.15 M NaCl, until the absorbance at 280 nm dropped to baseline. The rsT4's contaminant factor Bb remained adsorbed on the column due to its larger number of histidine residues and consequent stronger binding affinity to the immobilized copper.

This procedure afforded a stable rsT4 preparation of approximately 95% purity.

FINAL PURIFICATION OF rsT4 BY SIZE EXCLUSION CHROMATOGRAPHY

We added ammonium sulfate (472 g/L) to the rsT4-containing pool from the IMAC column. We collected the resulting precipitate containing the rsT4 by centrifugation (10,000 rpm at 4°C for 1 hour) and resolubilized the precipitate in 0.5% (w/v) glycine, pH 7.0, at 10 mg/ml. The resulting solution was applied to a 25 L Sephacryl S100 HR™ gel column, at 4°C at a flow rate for the column of 83 ml/min. We collected and pooled the central rsT4 peak, detected by absorbance at 280 nm, to obtain a protein of 98% purity. The elution buffer from the previous separation step was also exchanged in this final step for the buffer used in formulation.

We sterilized the pooled fraction by filtration on a YM100™ ultrafiltration membrane (Amicon, Danvers, MA), then concentrated the filtrate to 5 mg/ml by ultrafiltration on a PM-10™ filter

(Amicon, Danvers, MA). We adjusted this preparation to 5% (w/v) mannitol to yield an injectable composition, which was then freeze-dried in vials.

In the foregoing concentration and size exclusion step, we observed that the use of glycine retarded elution of the rsT4 from the column, giving it a slightly smaller apparent molecular weight. We substituted PBS for redissolving and eluting the rsT4, and this phenomenon was eliminated. For this reason, PBS is preferred for the final stage of rsT4 purification according to this embodiment, however in the purification of other proteins it is not expected that the use of glycine will have the same effect.

Example 2

An rsT4 sample was purified following the general procedure of Example 1, except that the buffers used on the IMAC column included 0.3 M NaCl (rather than 0.15 M NaCl), the elution buffer was 0.3 M Tris·HCl, and as an additional step the column was washed with a 0.4 M Tris·HCl buffer.

As a result of this procedure, a stable rsT4 preparation of approximately 95% purity was obtained. For the final concentration, and the size-exclusion step, the precipitated rsT4 was taken up in phosphate buffer (without glycine), pH 7.4, at 10 mg/ml and applied to the S100 gel column. The protein collected from the gel column was found to have a purity of 98%. The protein was subsequently sterilized and formulated into an injectable composition as in Example 1.

While the process of the invention has been described with reference to the separation of a particularly desirable protein, rsT4, the process will be suitable for many other proteins containing surface metal-binding amino acid residues such as histidine

and/or cysteine. Such proteins include, e.g., other soluble T4 proteins, as well as human serum proteins (such as IgG, haptoglobin, hemopexin, Gc-globulin, C1q, C3, C4), human ceruloplasmin, Dolichos biflorus lectin, zinc-inhibited Tyr(P) phosphatases, phenolase, carboxypeptidase isoenzymes, human copper-zinc superoxide dismutase, nucleoside diphosphatase, leukocyte interferon, fibroblast interferon, immune interferon, lactoferrin, human plasma $alpha_2$-SH glycoprotein, $alpha_2$-macroglobulin, $alpha_1$-antitrypsin, plasminogen activator, gastrointestinal polypeptides, pepsin, human and bovine serum albumin, granule proteins from granulocytes and lysozymes, non-histone proteins, human fibrinogen, human serum transferrin, human lymphotoxin, calmodulin, protein A, avidin, myoglobins, somatomedins, human growth hormone, transforming growth factors, platelet-derived growth factor, alpha-human atrial natriuetic polypeptide, cardiodilatin, and others. In addition, although in the specific examples of this disclosure column chromatography is described, batch methods could also be used. The process recited herein will also be useful in the purification of other soluble proteins derived from membrane-bound proteins, i.e., by the cloning of a gene coding for the extracellular region of the protein or by other techniques. All such purifications are within the intended scope of this invention as defined by the appended claims.

CLAIMS

We Claim:

1. A process for purifying a protein having surface metal-binding amino acid residues comprising the steps:

(a) contacting a solution containing the protein with an immobilized metal affinity chromatography (IMAC) resin, said resin comprising a matrix resin linked to a bidentate chelator bound to divalent metal ions, in a binding buffer containing salt and a weak ligand for said metal ions; and

(b) selectively eluting the protein using a buffer containing salt and a higher concentration of the weak ligand than in the binding buffer.

2. The process according to claim 1 wherein prior to step (a) the protein-containing solution is partially purified by dialysis, ultrafiltration, density-gradient centrifugation, molecular sieve chromatography, electrophoresis, ion-exchange chromatography, affinity chromatography, hydrophobic interaction chromatography, ammonium sulfate precipitation, or combinations thereof.

3. A process according to claim 1, wherein the weak ligand is Tris.

4. A process according to claim 3, wherein the binding buffer is 0.01-0.1 M Tris·HCl buffer.

5. A process according to claim 3, wherein the elution buffer is 0.1-0.5 M Tris·HCl buffer.

6. A process according to claim 3, wherein the binding buffer is about 0.02 M Tris·HCl and the elution buffer is about 0.3 M Tris·HCl buffer.

7. A process according to claim 6, wherein the binding buffer contains about 0.15 M NaCl, and the elution buffer also contains about 0.15 M NaCl.

8. The process according to claim 1 wherein the protein is recombinant soluble T4 which has a primary structure defined by the amino acid sequence depicted in Figure 2.

9. The process according to claim 1 wherein the divalent metal ion is Cu^{2+}.

10. The process according to claim 9 wherein the bidentate chelator is iminodiacetic acid (IDA).

11. The process according to claim 10 wherein the Cu^{2+} ion is chelated by the IDA and immobilized on an agarose resin at about pH 5 or higher.

12. The process according to claim 1 wherein the IMAC resin is washed with 0.02 M Tris·HCl buffer at pH 7.5, containing 0.15 M NaCl, following contacting of the sample containing the protein with the resin.

13. The process according to claim 1 wherein the proteins adsorbed on the IMAC resin are selectively eluted with Tris·HCl buffer at a concentration of about 0.3 M.

14. The process according to claim 13 wherein proteins with fewer metal-binding residues are eluted from the column first and are thus isolated.

15. The process according to claim 1, wherein the protein having surface metal-binding amino acid residues is selected from the group consisting of soluble T4, IgG, haptoglobin, hemopexin, Gc-globulin, C1q, C3, C4, human ceruloplasmin, Dolichos biflorus lectin, zinc-inhibited Tyr(P) phosphatases, phenolase, carboxypeptidase isoenzymes, human copper-zinc superoxide dismutase, nucleoside diphosphatase, leukocyte interferon, fibroblast interferon, immune interferon, lactoferrin, human plasma $alpha_2$-SH glycoprotein, $alpha_2$-macroglobulin, $alpha_1$-antitrypsin, plasminogen activator, gastrointestinal polypeptides, pepsin, human and bovine serum albumin, granule proteins from granulocytes and lysozymes, non-histone proteins, human fibrinogen, human serum transferrin, human lymphotoxin, calmodulin, protein A, avidin, myoglobins, somatomedins, human growth hormone, transforming growth factors, platelet-derived growth factor, alpha-human atrial natriuetic polypeptide, and cardiodilatin.

16. A process for purifying a recombinant soluble T4 protein (rsT4), comprising the steps of: (1) contacting culture medium containing rsT4, filtered free of the source cells which produced the rsT4, with a cationic exchange resin; (2) eluting the adsorbed proteins from the resin on the basis of their net charge; (3) applying the fraction or fractions of eluate from the cationic exchange resin containing the rsT4 to an anionic exchange resin; (4) applying the

wash fraction or fractions from step (3) containing rsT4 to an immobilized metal affinity chromatography resin using a binding buffer containing a low concentration of a weak ligand for the metal ion of the immobilized metal affinity chromatography resin; and (5) eluting the rsT4 using as an eluant a buffer having a higher concentration of said weak ligand.

17. The process according to claim 16, further comprising the steps: (6) concentrating the eluate of step (5) containing rsT4 by ammonium sulfate precipitation; and (7) solubilizing the precipitate of step (6) and applying the solution to a size exclusion chromatography resin.

18. The process according to claim 17 wherein, in the final step (7), the rsT4-containing precipitate is solubilized and subjected to size exclusion chromatography in a formulation buffer.

19. A process according to claim 18, wherein the weak ligand is Tris.

20. A process according to claim 19, wherein the binding buffer is 0.01-0.1 M Tris·HCl buffer.

21. A process according to claim 20, wherein the elution buffer is 0.1-0.5 M Tris·HCl buffer.

22. A process according to claim 19, wherein the binding buffer is about 0.02 M Tris·HCl and the elution buffer is about 0.3 M Tris·HCl buffer.

23. A process according to claim 21, wherein the binding buffer contains about 0.15 M NaCl, and the elution buffer also contains about 0.15 M NaCl.

24. The process according to claim 18, wherein said recombinant soluble T4 has a primary structure defined by the amino acid sequence depicted in Figure 2.

25. The process according to claim 18, wherein the divalent metal ion is Cu^{2+}.

26. The process according to claim 25, wherein the bidentate chelator is iminodiacetic acid (IDA).

27. The process according to claim 26, wherein the Cu^{2+} ion is chelated by the IDA and immobilized on an agarose resin.

28. The process according to claim 18, wherein the IMAC resin is washed with 0.02 M Tris·HCl buffer at pH 7.5, containing 0.15 M NaCl, following contacting of the sample containing the rsT4 with the IMAC resin.

29. The process according to claim 18, wherein the proteins adsorbed on the IMAC resin are selectively eluted with Tris·HCl buffer at a concentration of about 0.3 M.

30. A stable, homogeneous preparation of recombinant soluble T4 protein in native conformation produced according to the process of claim 19.

31. A preparation of rsT4 according to claim 30 which comprises recombinant soluble T4 protein of greater than 98% purity.

FIG. 1

Amino Acid Sequence of Transmembrane T4 (CD4) (Maddon et al.)

MetAsnArgGlyValProPheArgHisLeuLeuLeuValLeuGlnLeuAlaLeuLeuPro 20

AlaAlaThrGlnGlyAsnLysValValLeuGlyLysLysGlyAspThrValGluLeuThr 40

CysThrAlaSerGlnLysLysSerIleGlnPheHisTrpLysAsnSerAsnGlnIleLys 60

IleLeuGlyAsnGlnGlySerPheLeuThrLysGlyProSerLysLeuAsnAspArgAla 80

AspSerArgArgSerLeuTrpAspGlnGlyAsnPheProLeuIleIleLysAsnLeuLys 100

IleGluAspSerAspThrTyrIleCysGluValGluAspGlnLysGluGluValGlnLeu 120

LeuValPheGlyLeuThrAlaAsnSerAspThrHisLeuLeuGlnGlyGlnSerLeuThr 140

LeuThrLeuGluSerProProGlySerSerProSerValGlnCysArgSerProArgGly 160

LysAsnIleGlnGlyGlyLysThrLeuSerValSerGlnLeuGluLeuGlnAspSerGly 180

ThrTrpThrCysThrValLeuGlnAsnGlnLysLysValGluPheLysIleAspIleVal 200

ValLeuAlaPheGlnLysAlaSerSerIleValTyrLysLysGluGlyGluGlnValGlu 220

PheSerPheProLeuAlaPheThrValGluLysLeuThrGlySerGlyGluLeuTrpTrp 240

GlnAlaGluArgAlaSerSerSerLysSerTrpIleThrPheAspLeuLysAsnLysGlu 260

ValSerValLysArgValThrGlnAspProLysLeuGlnMetGlyLysLysLeuProLeu 280

HisLeuThrLeuProGlnAlaLeuProGlnTyrAlaGlySerGlyAsnLeuThrLeuAla 300

LeuGluAlaLysThrGlyLysLeuHisGlnGluValAsnLeuValValMetArgAlaThr 320

GlnLeuGlnLysAsnLeuThrCysGluValTrpGlyProThrSerProLysLeuMetLeu 340

SerLeuLysLeuGluAsnLysGluAlaLysValSerLysArgGluLysAlaValTrpVal 360

LeuAsnProGluAlaGlyMetTrpGlnCysLeuLeuSerAspSerGlyGlnValLeuLeu 380

GluSerAsnIleLysValLeuProThrTrpSerThrProValGlnProMetAlaLeuIle 400

ValLeuGlyGlyValAlaGlyLeuLeuLeuPheIleGlyLeuGlyIlePhePheCysVal 420

ArgCysArgHisArgArgArgGlnAlaGluArgMetSerGlnIleLysArgLeuLeuSer 440

GluLysLysThrCysGlnCysProHisArgPheGlnLysThrCysSerProIle 458

FIG. 2

Amino Acid Sequence of rsT4 Protein

```
AsnLysValValLeuGlyLysLysGlyAspThrValGluLeuThrCysThrAlaSerGln  20
LysLysSerIleGlnPheHisTrpLysAsnSerAsnGlnIleLysIleLeuGlyAsnGln  40
GlySerPheLeuThrLysGlyProSerLysLeuAsnAspArgAlaAspSerArgArgSer  60
LeuTrpAspGlnGlyAsnPheProLeuIleIleLysAsnLeuLysIleGluAspSerAsp  80
ThrTyrIleCysGluValGluAspGlnLysGluGluValGlnLeuLeuValPheGlyLeu 100
ThrAlaAsnSerAspThrHisLeuLeuGlnGlyGlnSerLeuThrLeuThrLeuGluSer 120
ProProGlySerSerProSerValGlnCysArgSerProArgGlyLysAsnIleGlnGly 140
GlyLysThrLeuSerValSerGlnLeuGluLeuGlnAspSerGlyThrTrpThrCysThr 160
ValLeuGlnAsnGlnLysLysValGluPheLysIleAspIleValValLeuAlaPheGln 180
LysAlaSerSerIleValTyrLysLysGluGlyGluGlnValGluPheSerPheProLeu 200
AlaPheThrValGluLysLeuThrGlySerGlyGluLeuTrpTrpGlnAlaGluArgAla 220
SerSerSerLysSerTrpIleThrPheAspLeuLysAsnLysGluValSerValLysArg 240
ValThrGlnAspProLysLeuGlnMetGlyLysLysLeuProLeuHisLeuThrLeuPro 260
GlnAlaLeuProGlnTyrAlaGlySerGlyAsnLeuThrLeuAlaLeuGluAlaLysThr 280
GlyLysLeuHisGlnGluValAsnLeuValValMetArgAlaThrGlnLeuGlnLysAsn 300
LeuThrCysGluValTrpGlyProThrSerProLysLeuMetLeuSerLeuLysLeuGlu 320
AsnLysGluAlaLysValSerLysArgGluLysAlaValTrpValLeuAsnProGluAla 340
GlyMetTrpGlnCysLeuLeuSerAspSerGlyGlnValLeuLeuGluSerAsnIleLys 360
ValLeuProThrTrpSerThrProValGlnProMetAlaLeuIle               375
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FIG. 3

Amino Acid Sequence of Fragment Bb from Human Complement Factor B

LysIleValLeuAspProSerGlySerMetAsnIleTyrLeuValLeuAspGlySerAsp 20

SerIleGlyAlaSerAsnPheThrGlyAlaLysLysCysLeuValAsnLeuThrGluLys 40

ValAlaSerTyrGlyValLysProArgTyrGlyLeuValThrTyrAlaThrTyrProLys 60

IleTrpValLysValSerGluAlaAspSerSerAsnAlaAspTrpValThrLysGlnLeu 80

AsnGluIleAsnTyrGluAspHisLysLeuLysSerGlyThrAsnThrLysLysAlaLeu 100

GlnAlaValTyrSerMetMetSerTrpProAspAspValProProGluGlyTrpAsnArg 120

ThrArgHisValIleIleLeuMetThrAspGlyLeuHisAsnMetGlyGlyAspProIle 140

ThrValIleAspGluIleArgAspLeuLeuTyrIleGlyLysAspArgLysAsnProArg 160

GluAspTyrLeuAspValTyrValPheGlyValGlyProLeuValAsnGlnValAsnIle 180

AsnAlaLeuAlaSerLysLysAspAsnGluGlnHisValPheLysValLysAspMetGlu 200

AsnLeuGluAspValPheTyrGlnMetIleAspGluSerGlnSerLeuSerLeuCysGly 220

MetValTrpGluHisArgLysGlyThrAspTyrHisLysGlnProTrpGlnAlaLysIle 240

SerValIleArgProSerLysGlyHisGluSerCysMetGlyAlaValValSerGluTyr 260

PheValLeuThrAlaAlaHisCysPheThrValAspAspLysGluHisSerIleLysVal 280

SerValGlyGlyGluLysArgAspLeuGluIleGluValValLeuPheHisProAsnTyr 300

AsnIleAsnGlyLysLysGluAlaGlyIleProGluPheTyrAspTyrAspValAlaLeu 320

IleLysLeuLysAsnLysLeuLysTyrGlyGlnThrIleArgProIleCysLeuProCys 340

ThrGluGlyThrThrArgAlaLeuArgLeuProProThrThrThrCysGlnGlnGlnLys 360

GluGluLeuLeuProAlaGlnAspIleLysAlaLeuPheValSerGluGluGluLysLys 380

LeuThrArgLysGluValTyrIleLysAsnGlyAspLysLysGlySerCysGluArgAsp 400

AlaGlnTyrAlaProGlyTyrAspLysValLysAspIleSerGluValValThrProArg 420

PheLeuCysThrGlyGlyValSerProTyrAlaAspProAsnThrCysArgGlyAspSer 440

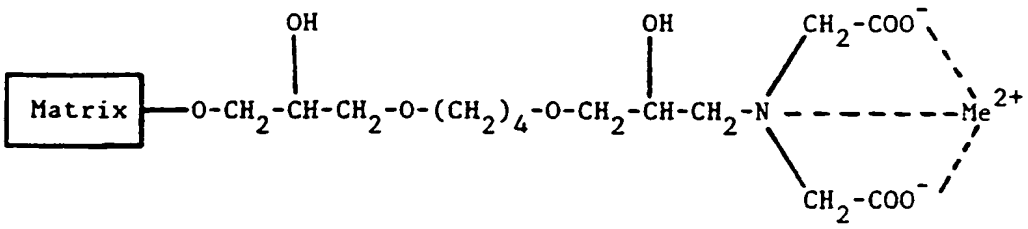
GlyGlyProLeuIleValHisLysArgSerArgPheIleGlnValGlyValIleSerTrp 460

GlyValValAspValCysLysAsnGlnLysArgGlnLysGlnValProAlaHisAlaArg 480

AspPheHisIleAsnLeuPheGlnValLeuProTrpLeuLysGluLysLeuGlnAspGlu 500

AspLeuGlyPheLeu 505

FIG. 4



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/01991

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C07K 3/14,3/18,3/20,3/22,3/24,3/26,3/28, 15/00, 15/14, 13/00

U.S. CL.: 530/350,395,412,413,414,415,416,417,418,420,427

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	530/350,395,412,413,414,415,416,417,418,420,427

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

APS and DIALOG Files 5, 35, 72, 135, 350, 351, 357, 399 searched for metal binding in purification, Tris etc. and T4 and CD4.

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	Trends in Biotechnology, Published 1985, "Purification of proteins by IMAC", (Sulkowski), Vol. 3	1,9-11,14 and 15 2-7,12 and 13
Y	Biochemica et Biophysica Acta, Published 1979, "Purification and Partial Characterization of Plasminogen Activator from Human Uterine Tissue", (Rijken et al.), Vol. 580, pages 140-153. See pages 140, 144, 145 and 150.	1-15
Y	Journal of Chromatography, Published 1980, "Metal Chelate Affinity Chromatography: I. Influence of Various Parameters on the Retention of Nucleotides and Related Compounds", (Hubert et al.), Vol. 198, pages 247-255. See pages 249-251.	1-15
Y	Journal of Chromatography, Published 1986, "High-Performance Immobilized-Metal Affinity Chromatography of Proteins on Iminodiacetic Acid Silica-Based Bonded Phases", (Figueroa et al.), Vol. 371, pages 335-352. See pages 335 and 343.	1-15

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
05 JULY 1990	28 AUG 1990
International Searching Authority	**Signature of Authorized Officer**
ISA/US	KEITH C. FURMAN

Form PCT/ISA/210 (second sheet) (Rev. 11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Biochemistry, Published 1983, "Immobilized Metal Ion Affinity Adsorption and Immobilized Metal Ion Affinity Chromatography of Biomaterials. Serum Protein Affinities for Gel-Immobilized Iron and Nickel Ions", (Porath et al.), Vol. 22, pages 1621-1630. See whole publication.	1-29

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	Nature, Published 07 January 1988, "HIV Infection is Blocked in vitro by Recombinant Soluble CD4", (Fisher et al.), Vol. 331, pages 76-78. See page 78.	30,31
X	Nature, Published 07 January 1988, "A Soluble CD4 Protein Selectively Inhibits HIV Replication and Syncytium Formation", (Hussey et al.), Vol. 331, pages 78-81. See page 79.	30,31

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.